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Previous animal models of heat stress have been compromised by methodologies, such as restraint and anesthesia that confound our understanding of the core temperature (Tc) responses elicited by heat stress. Using biotelemetry, we developed a heat stress model to examine Tc responses in conscious, unrestrained C57BL/6J male mice. Prior to heat stress, mice were acclimated for > 4 weeks to Ta of 25oC. Mice were exposed to ambient temperature (Ta) of 39.5± 0.2oC, in the absence of food and water, until they reached maximum Tc (Tc, Max) of 42.4 (N= 11), 42.7 (N= 12), or 43.0oC (N= 11), defined as mild, moderate and extreme heat stress, respectively. Heat stress induced ~13% BW loss that did not differ by final group Tc, whereas survival rate was affected by final Tc (100% at 42.4oC; 92% at 42.7oC; 46% at 43oC). Hypothermia (Tc< 34.5oC) developed following heat stress with the depth and duration of hypothermia significantly enhanced in the moderate and extreme compared to the mild group. Regardless of heat stress severity, every mouse that transitioned out of hypothermia (survivors only) developed a virtually identical elevation in Tc the following day, but not night, compared to non-heated controls. To test the effect of recovery Ta, a group of mice (N= 5) were acclimated for > 4 weeks and recovered at Ta of 30oC following moderate heat stress. Recovery at 30oC resulted in 0% survival within ~2h following cessation of heat stress. Using biotelemetry to monitor Tc in the unrestrained mouse, we have shown that recovery from acute heat stress is associated with prolonged hypothermia followed by an elevation in daytime Tc that is dependent on Ta. These thermoregulatory responses to heat stress are key biomarkers that may provide insight into heat stroke pathophysiology.

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Heat stress induces a biphasic thermoregulatory response in mice

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fever; hypothermia; hyperthermia; dehydration; body temperature

THE ADVERSE EFFECTS OF HEAT stress have been noted as far back as 3000 BC. Fever and madness were correlated with the summer appearance of the Dog Star, Sirius in the Canis Major constellation, from which stems the phrase "dog days" of summer and the medical term siriasis, a general descriptive for heat illness (10). Today, heat injury is not only a sports (2) and military (6) medical problem, but, as exemplified by the recent high death toll in France (8), is also a public health issue that may escalate with global warming (23). Animal models that permit identification of the mechanisms that contribute to heat injury or support heat stress recovery are required to address this potential threat.

From rodent models, the core temperature (T_c) profile during heat stress progression has been characterized as a triphasic pattern, with an initial linear T_c increase, a subsequent equilibrium plateau, and a final rapid progression to a lethal T_c (26, 33, 34). Although interindividual variability in rodent thermal resistance has been recognized (26, 34), the impact of methodological confounders on experimental heat stress variability has not been clearly delineated. Current rodent models are compromised by confounders such as the use of rectal temperature probes, restraint, or anesthesia (14, 26, 27, 31, 33, 34) that influence the thermoregulatory profiles generated during and after heat stress. In rodents, the behavioral spreading of saliva on the ventral body surfaces is an essential mechanism for evaporative heat loss that is prevented under restrained conditions. Anesthesia induces hypothermia through the inhibition of several thermoregulatory mechanisms (13, 28, 30), thus making the study of T_c inappropriate under these conditions. The insertion of rectal probes or attachment of thermocouples induces T_c changes, which are difficult to distinguish from hyperthermia in response to an environmental heat load. Thus methodological features of earlier studies have disturbed normal behavior and physiological mechanisms as animals are challenged with controlling T_c in a hot environment, thus producing an unnatural T_c response to the heat insult. The recognition of these study deficiencies leaves in question the thermoregulatory profile of rodents during and after heat stress.

Biotelemetry permits the remote sensing of T_c in conscious, freely moving animals throughout the circadian cycle. As such, it is a powerful technique for the study of thermoregulation in rodent heat stress models because it eliminates the previously described confounders from influencing the study outcome. We used biotelemetry to determine the T_c responses of mice during progression and recovery from heat stress of varying severity. Our main objective was to determine the responses elicited in conscious, unrestrained, freely moving mice that were able to use their behavioral and autonomic thermoeffectors to survive and respond to prolonged exposure to a hot environment. Because rodent heat stress responses through consecutive circadian cycles have not been previously analyzed, we extended our thermoregulatory analysis through 72 h of undisturbed recovery. Furthermore, due to the profound impact of ambient temperature (T_a) on T_c of small rodents, we tested the hypothesis that the thermoregulatory profile displayed during heat stress recovery would be dependent on T_a. For this aspect of the study, mice were housed at a T_a below (25 ± 2°C) or within (30 ± 2°C) the thermoneutral zone (TNZ) for this species (11). Finally, we determined the variability in thermal tolerance of mice heated to different maximum T_c ($T_{c,max}$), since previous reports suggested a range as large as 40.4–46.0°C, which may have been a consequence of the previously described methodological confounders in use (14, 26, 27, 31, 33, 34).

We describe here a biphasic thermoregulatory recovery profile of mice that is dependent on T_a and heat stress severity.

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Through an analysis of T_c, we provide a basis from which the full range of factors that influence morbidity and survival might be studied, employing humane endpoints.

MATERIALS AND METHODS

Animals. Seventy-eight adult C57BL/6J male mice (Jackson Laboratories, Bar Harbor, ME) weighing 25.8 ± 0.6 g (~3 mo of age) were used. Mice were individually housed in Nalgene polycarbonate cages (11.5 in. × 7.5 in. × 5 in.) fitted with Hepa-filter cage tops and wood-chip bedding (Pro-Chip, PWI). Rodent laboratory chow (Harlan Teklad, LM-485, Madison, WI) and water were provided ad libitum as mice were acclimated to T_a of 25 ± 2°C (A25 group) or 30 ± 2°C (A30 group) for a minimum of 4 wk before experimentation (12:12-h light-dark cycle; lights on at 0600). These T_a values were chosen for acclimation because they represent common T_a used in thermoregulation studies in mice and are below and within the TNZ of this species, respectively (11, 17, 19–22, 33). In conducting research using animals, we adhered to the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council; all mice were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility. All procedures received Institutional Animal Care and Use Committee approval before experimentation.

Biotelemetry measurements. T_c (±0.1°C) was continuously monitored by biotelemetry in mice implanted with a transmitter (Dataquest A.R.T. system, Data Sciences International, St. Paul, MN). Briefly, each animal was anesthetized with isoflurane, and a temperature-sensitive transmitter (model TA10TA-F20) was surgically implanted intra-abdominally by aseptic techniques. Frequency of the emitted transmitter signal is proportional to T_c. An antenna placed under each animal's cage received the transmitter signal and converted it to T_c using predetermined calibration values. T_c was collected at 1-min intervals. Transmitter weights were ~3.7 g, which represented ~14% of mouse body weight (BW). To be included in experimentation, mice had to regain presurgical BW and establish a stable circadian T_c rhythm after transmitter surgery. At least 2 wk were required to satisfy both of these conditions, as previously described in this species (21).

Incubator acclimation before heat stress. Before establishing our heat stress protocol, we determined the T_c response of mice after their placement into the incubator that was to be used for heat exposure (model 3950, Therma Forma, Marietta, OH). We observed that initial exposure to the incubator, which we considered a novel environment, at housing T_a of 25°C or 30°C induced stress-induced hyperthermia that required ~3 h for recovery to baseline T_c (data not shown). This stress-induced hyperthermia was effectively eliminated after a 24-h acclimation to the incubator at the two housing T_a (data not shown). Furthermore, mice exposed to the incubator for 24 h showed similar weighing-induced hyperthermia as control mice housed on animal racks outside of the incubator at the same T_a. Thus, for all experiments, mice that were assigned to the heat stress condition remained in their home cages with ad libitum food and water and were placed into an incubator set at T_a of 25°C or 30°C for 24 h before experimentation. Mice that served as nonheated controls remained in their home cages with bedding and ad libitum food and water in their original cage location during experimentation. Thus nonheated mice were not tested in the incubator environment.

Heating protocol. Each A25 mouse was exposed to both a control and heat stress condition, using a counterbalance design. This was not possible in the A30 condition, due to a significant reduction in survival rate compared with the A25 group (see details below). After A25 mice were exposed to the first condition, full BW recovery and a stable circadian T_c rhythm was required before inclusion in the next condition. At least 1 wk between experimental conditions was required for BW recovery (data not shown). Approximately 24 h before heat stress, each mouse was randomly preassigned into a control or heat stress group. At this time, cage filter tops were removed from all cages to facilitate air circulation during experimentation, and heat stress mice were placed into the incubator for 24-h acclimation, whereas control mice remained in their original cage location. The following day between 0800 and 0900 (time of baseline T_c of <36.0°C), each control and heat stress mouse was weighed, and food and water were removed from cages. Mice were heated in the same cage, with wood-chip bedding, as they were housed. To initiate heat stress, the incubator T_a was increased to 39.5 ± 0.2°C (T_{hs}); incubator T_a gradually increased until it reached T_{hs} within ~1 h. In initial experiments, we observed heat stress in mice to determine the $T_{c,max}$ at which mice retained normal locomotor activity (data not shown) and survived. This was termed mild heat stress ($T_{c,max}$ = 42.4°C; n = 11). From the mild $T_{c,max}$, increases of 0.3 and 0.6°C were used to designate moderate (42.7°C; n = 12) and extreme (43.0°C; n = 11) heat stress groups. Mice from each mild, moderate, or extreme heat stress group remained in the incubator until their preassigned $T_{c,max}$ was attained. After attainment of $T_{c,max}$, mice were removed from the incubator, weighed, and returned to T_a of 25°C (A25 group) or 30°C (A30 group) with food and water in the same cage in which they were heat stressed. At this time, respective control mice were weighed and provided with food and water. T_c was monitored during 72 h of undisturbed recovery.

Four shipments of mice were required to obtain sufficient numbers for statistical analysis of T_c responses between the control and heat stress conditions. To ensure that BW and age did not differ between animal populations, at least one mouse was tested in the control, mild, moderate, and extreme heat stress group each week.

Humane endpoints. Because of the unexpected decrease in survival rate in the extreme heat stress group and to minimize animal distress during recovery, we compared two aspects of the thermoregulatory profile between survivors and nonsurvivors, including *1*) lowest 1-h average T_c observed during recovery and *2*) the first time point post-heat stress that survivors demonstrated significant transition out of hypothermia compared with nonsurvivors. This post hoc analysis of the thermoregulatory profiles of survivors (n = 5) and nonsurvivors (n = 6) allowed us to define humane endpoints for the extreme heat stress group that could be employed in future experimentation with this model.

Influence of recovery T_a. To determine whether acclimation and recovery T_a influence heat stress responses, an additional group of C57BL/6J male mice were exposed to control (n = 5) or moderate heat stress (n = 5) after acclimation for >4 wk at T_a of 30°C (A30 condition). We chose 30°C for this experiment because this T_a is within the TNZ for this species (~28–32°C; Ref. 11) and is a housing T_a commonly used in thermoregulation studies in mice (17, 19, 20). This group was tested with the same heat stress protocol described above, with the exception that mice were housed at 30°C before and during recovery from heat stress. Because heat stress recovery at this T_a resulted in unexpected high mortality, this study was terminated after experimentation with five mice, when it was demonstrated that this group displayed post-heat stress T_c responses significantly different from the A25 group and when humane endpoints were identified. The high mortality precluded use of counterbalance design at this recovery T_a.

Dehydration. BW was corrected for transmitter weights and difference in pre- and post-BW used to determine dehydration. Each mouse was weighed on a top-loading balance accurate to ±0.1 g. BW was obtained immediately before the start of heat stress and after attainment of the preassigned $T_{c,max}$.

Heating calculations. Figure 1 provides graphical representation of the calculations that were performed to describe the thermoregulatory responses of heat stress mice. Time (in min) to $T_{c,max}$ represents total heat stress exposure time. Total thermal area (°C·min), which was used as an indication of thermal strain, was calculated as Σ of the time intervals (min) × 0.5 (°C above T_c = 39.5°C at the start of the

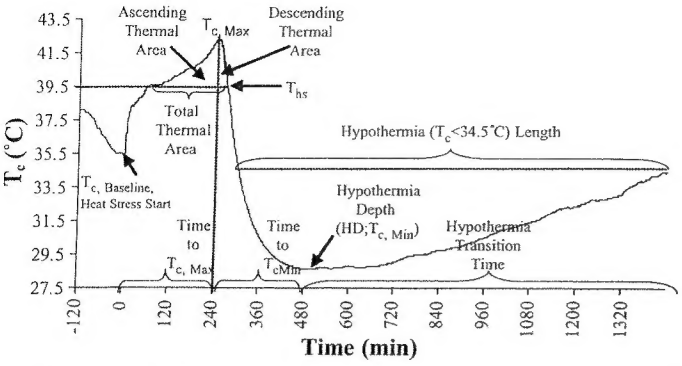

Fig. 1. Graphical representation of calculations used for description of thermoregulatory aspects of the heat stress profile in mice. Curve depicts a typical thermoregulatory profile from a heat-stressed mouse. Times are approximate. T_c, core body temperature; $T_{c,max}$, final T_c during heating; $T_{c,min}$, minimum T_c observed during recovery; $T_{c,baseline}$, T_c observed immediately before start of heat stress; T_{hs}, ambient temperature (T_a) used to induced heat stress (39.5°C); HD, hypothermia depth, calculated as lowest 1-h average T_c observed during recovery.

interval + °C above T_c = 39.5°C at the end of the interval). T_c = 39.5°C was used because it equaled T_{hs} and represented the point above which mice were able to radiate excess body heat to the environment (i.e., $T_c > T_{hs}$). The ascending (39.5°C to $T_{c,max}$) and descending ($T_{c,max}$ to 39.5°C) aspects were segregated to further describe thermal area. Hypothermia was defined as $T_c < 34.5$°C, which was the lowest T_c value observed in an undisturbed nonheated mouse before heat stress (data not shown). Minimum T_c ($T_{c,min}$; °C) represents the lowest 1-min T_c value observed during heat stress recovery. Time to reach $T_{c,min}$ (min) post-heat stress was calculated as the time from $T_{c,max}$ to $T_{c,min}$. Hypothermia depth (HD; °C) was the lowest 1-h average T_c during recovery. Hypothermia length (HL; min) was the total time $T_c < 34.5$°C during recovery. Hypothermia transition time (min) was the time to return from $T_{c,min}$ to T_c = 34.5°C.

Dehydration calculations. Estimation of the percent dehydration was accomplished as follows: [(pre-heat stress BW − post-heat stress BW)/pre-heat stress BW] × 100.

Survival. Survival was assessed by observing T_c when immediate visual observation could not be performed (e.g., during lights off). Nonsurvival was determined by observing a rapid decrease in T_c slope that exceeded that observed during all phases of hypothermia development in heat stress survivors. Once a rapid T_c slope was observed, nonsurvival was verified by visual inspection of the animal. When heat stress significantly decreased survival, only a minimum number of mice were used to define humane endpoints and to determine the influence of recovery T_a on the thermoregulatory profile.

Data analysis. Data are presented as means ± SE. With the exception of the A30 group, mice succumbing to heat stress before serving as a control were excluded (n = 3). T_c is presented as 1-min values, unless otherwise specified. Two-way ANOVA with Tukey's post hoc test determined main group and time effects. One-way ANOVA with Tukey's post hoc test determined group effects of heating time, thermal area, time to reach $T_{c,min}$, hypothermia transition time, HD, and HL. X^2 was used to analyze survival rates. For extreme heat stress survivor/nonsurvivor comparisons, we tested lowest hypothermia T_c post-heat stress for significant differences using Student's t-test. We analyzed recovery from hypothermia using repeated-measures ANOVA with Tukey's post hoc test. Significance was set at $P < 0.05$.

RESULTS

Baseline T_c. Circadian T_c profiles of A25 and A30 mice were similar with low daytime (12 h average of 36.1 ± 0.1°C) and high nighttime values (12 h average of 37.2 ± 0.1°C). Periodic awakenings during the daytime (inactive) or lights-on period were manifested as ultradian spikes in T_c (data not shown). These ultradian spikes were apparent in both A25 and A30 mice and occurred at different circadian times in each animal. To normalize baseline T_c and ensure that each mouse experienced the same total change in T_c during heat exposure, these ultradian spikes were monitored to confirm a quiescent baseline T_c of <36.0°C before the start of experimentation for all mice.

Heat stress thermoregulatory profiles in A25 mice. Figure 2 shows the 48-h T_c profile of A25 control and heat stress mice. All control mice (n = 34) showed virtually identical T_c rhythms during experimentation and are presented as one group. Because of interindividual variability in thermal tolerance, each heat stress mouse is presented individually for comparison to the control group. At *time 0*, control mice had a transient (~1 h) increase in T_c, indicative of weighing-induced hyperthermia. Weighing induced a similar response in heat stress mice, followed by a steep increase in T_c due to uncompensated environmental heat load as $T_{hs} > T_c$. Once $T_c > T_{hs}$, heat stress mice effectively dissipated body heat to the environment to decrease T_c slope, such that a plateau in T_c rise was discernable starting at ~45–60 min (Fig. 2). A second increase in T_c slope subsequently developed, indicating a breakdown of thermoregulatory control as $T_{c,max}$ was approached. In the absence of food and water, control mice maintained the normal baseline T_c from ~60 to 270 min, after which the second weighing-induced hyperthermia was evident (~270 min, Fig. 2). The time of the second weighing of control mice was matched to the attainment of $T_{c,max}$ of heat stress mice.

Time to reach $T_{c,max}$ did not differ significantly with increases in heat stress severity (Table 1) or between extreme heat stress survivors and nonsurvivors (data not shown). Total thermal area was significantly greater for the extreme heat stress group compared with the mild group only (Table 1). Although ascending thermal areas were similar, descending thermal areas were significantly elevated as heat stress severity increased (Table 1; ANOVA, $P < 0.05$).

Hypothermia post-heat stress in A25 mice. Within 1 h of recovery, A25 heat stress groups decreased T_c below 34.5°C and developed hypothermia (Fig. 2). Mice heated to 42.4°C transitioned from hypothermia during the first night post-heat stress (Fig. 2*A*). Mice heated to 42.7°C showed variability in hypothermia transition (Fig. 2*B*). Two mice did not complete transition until the second night, and one mouse sustained hypothermia throughout 48 h of recovery (Fig. 2*B*) and subsequently died (data not shown). Variability was also evident in hypothermia transition for extreme heat stress (Fig. 2*C*). Two mice succumbed immediately after removal from the heat, whereas four other nonsurvivors remained hypothermic <30 h.

Table 1 characterizes the hypothermia phase of heat stress survivors. Time to reach $T_{c,min}$ was significantly extended in the extreme compared with the mild and moderate heat stress groups (ANOVA, $P < 0.05$). Time to reach $T_{c,min}$ in survivors and nonsurvivors of the extreme group was virtually identical (data not shown). HD (the lowest 1-h average T_c) results for survivors of moderate and extreme heat stress were significantly lower than those for the mild heat stress group

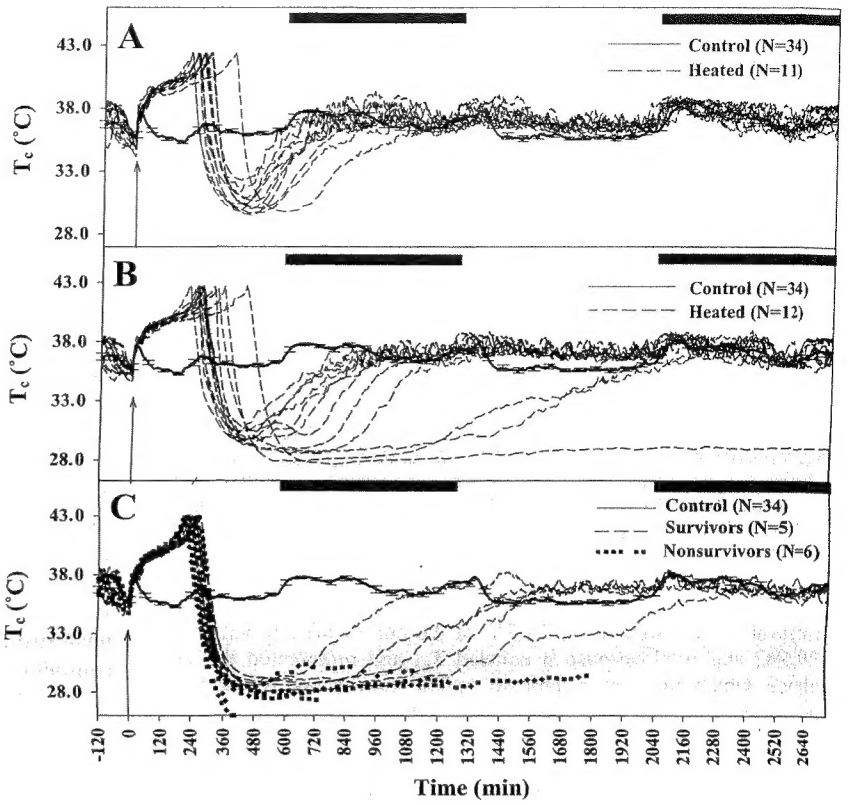

Fig. 2. Individual T_c responses of C57BL/6J male mice heated at $T_a = 39.5 \pm 0.2$°C to $T_{c,max}$ of 42.4 (mild; *A*), 42.7 (moderate; *B*), and 43.0°C (extreme; *C*) compared with mean responses of controls. Acclimation and recovery were at $T_a = 25 \pm 2$°C. Arrows at *time 0* indicate start of heat stress. Dark horizontal bars represent lights-off (active) period. Sample sizes (no. of mice) are shown in parentheses.

(ANOVA, $P < 0.05$; Table 1). HL (time $T_c < 34.5$°C) results were significantly greater in the moderate and extreme compared with the mild heat stress group (Table 1; ANOVA, $P < 0.05$). Hypothermia transition time was significantly prolonged in the extreme compared with the mild group (Table 1; ANOVA, $P < 0.05$). Irrespective of heat stress severity, any mouse that did not transition from hypothermia died.

T_c elevations post-heat stress in A25 mice. The day after heat stress, A25 control mice had a normal circadian T_c rhythm, indicating that the previous day of testing had not influenced circadian rhythmicity (Fig. 2). After transition from hypothermia, all A25 heat stress mice displayed a significant elevation of T_c compared with controls (ANOVA, $P < 0.05$; Fig. 2). Those animals with a prolonged hypothermia transition phase, which extended through the day after heat stress, developed a T_c elevation compared with controls on the third day (data not shown). Thus hypothermia was always followed by a phase of T_c elevation in heat stress survivors.

Figure 3 illustrates the T_c elevation of A25 mice that transitioned from hypothermia the day after heat stress. Mild and moderate heat stress groups that transitioned before 0600 h displayed a significantly elevated T_c throughout the daytime compared with control mice. The extreme heat stress group had a delayed transition but subsequently developed T_c elevations that did not differ from those in the other heat stress groups by 1000 h. From 1000–1745 h, the T_c elevation was virtually indistinguishable among heat stress groups (37.1 ± 0.1°C) compared with nonheated controls (35.8 ± 0.2°C). Although

Table 1. *Responses of heat-stressed C57BL/6J male mice*

		A25 Group			A30 Group
T_c Response	Control (n = 39)	42.4°C (n = 11)	42.7°C (n = 12)	43.0°C (n = 5)	42.7°C (n = 5)
Time to reach $T_{c,max}$, min		265±13	275±11	252±7	240±17
Time to reach $T_{c,min}$, min		179±7[a]	254±35[b]	394±68[a,b]	
Hypothermia depth, °C		30.5±0.3[e,f]	29.4±0.39[e]	28.7±0.2[f]	
Hypothermia length, min		341±24[g,h]	752±161[g]	1,316±308[h]	
Hypothermia transition time, min		183±22[i]	433±111	766±179[i]	
Survival, %	100	100[j]	92[k]	46[j,k]	0
Body weight loss (dehydration), %	2	13	13	12	12
Total thermal area, °C·min		275.0±13.5[l]	331.0±21.5	356.1±11.4[l]	388.4±20.5
Ascending thermal area, °C·min		246.8±13.5	290.7±20.0	299.8±10.2	319.9±18.0
Descending thermal area, °C·min		28.1±0.7[m]	40.3±3.1[m]	56.3±4.0[m]	68.5±5.4

Values are means ± SE; sample sizes (n) are indicated in parentheses. Values were calculated for survivors of the 72-h recovery period only. A25 group, mice housed at ambient temperature of 25 ± 2°C; A30 group, mice housed at ambient temperature of 30 ± 2°C; T_c, core body temperature; $T_{c,max}$, maximum T_c during heating; $T_{c,min}$, minimum T_c during recovery. Significance, set at $P < 0.05$, between A25 heat stress groups is depicted by values with similar letters.

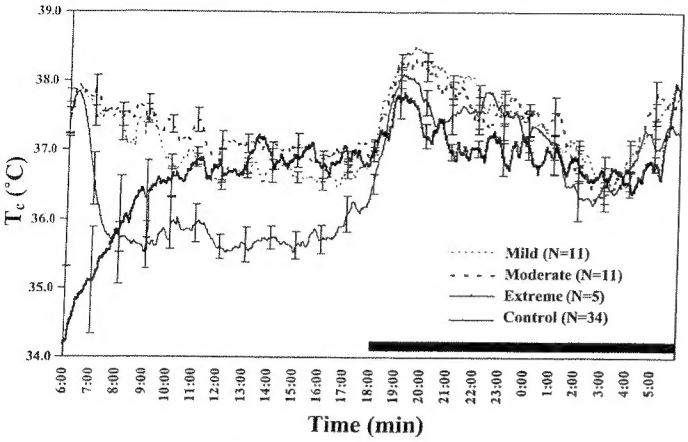

Fig. 3. T_c 24–48 h after heat stress in C57BL/6J male mice during recovery at T_a = 25 ± 2°C. Data represent survivors only. Sample sizes (no. of mice) are shown in parentheses. Dark horizontal bar represents lights-off (active) period.

the heat stress groups sustained higher daytime T_c profiles compared with control mice, nighttime profiles were similar.

Influence of acclimation and recovery T_a. Figure 4 shows the effects of 30°C acclimation and recovery T_a on moderate heat stress outcome. Because the circadian T_c profile of A30 mice was virtually identical to that observed in A25 mice with regard to resting (daytime; 36.1 ± 0.2°C) and active (nighttime; 37.2 ± 0.1°C) T_c values, experimentation was begun at the same baseline T_c (<36.0°C) as with A25 mice. The A30 heating profiles were similar to those in A25 mice in that a hyperthermia plateau was apparent before $T_{c,max}$ = 42.7°C was reached (Fig. 2 vs. Fig. 4). A30 mice had similar total and ascending thermal areas but a significantly greater descending thermal area compared with the A25 group (Table 1; ANOVA, $P < 0.001$). After removal from the heat and placement at 30°C, mice were unable to effectively dissipate heat to the environment and succumbed at various time points during recovery. Unlike A25 groups that recovered, all A30 mice succumbed within 2.3 ± 1.2 h post-heat stress, which was before they became hypothermic (i.e., T_c < 34.5°C). Thus hypothermia and elevations in T_c the day after heat stress were not observed in A30 mice.

Dehydration as reflected by BW loss. BW (25.8 ± 0.6g) and age (92 ± 5 days) were similar among A25 and A30 groups. A25 and A30 control mice lost 2.0% and 2.5% BW, respectively, which did not represent a significant difference between groups. Presumably, this BW loss was due to an absence of food and/or water during testing and weighing-induced stress. A25 and A30 heat stress mice also had similar BW loss (~13%) (Table 1). In all cases, heat stress induced significantly greater BW loss than the control condition, but differences were not detected between heat stress groups of varying severity.

Survival. Table 1 shows survival of A25 and A30 heat stress groups. All nonheated control mice survived the 72-h observation period. Mild heat stress induced no fatalities. A25 and A30 moderate heat stress groups had a 92% and 0% survival rate, respectively (X^2, $P = 0.001$). Survival rate for extreme heat stress was 46% at 72 h.

Humane endpoints for extreme heat stress mice. HD was significantly lower for nonsurvivors (26.6 ± 0.8°C) than for survivors (28.6 ± 0.3°C; Student's *t*-test, $P = 0.03$) of the extreme heat stress group. The first time point post-heat stress that survivors demonstrated significant transition out of hypothermia compared with nonsurvivors was at 765 min (Fig. 5; ANOVA, $P < 0.05$).

DISCUSSION

Previous animal models of heat stress have been compromised by methodologies, such as restraint and anesthesia, that alter thermoregulatory control mechanisms and confound our present understanding of the T_c responses elicited during progression and recovery from heat stress. Using biotelemetry to measure T_c, we developed a model to minimize experimental confounders and to define the recovery profiles of mild, moderate, and extreme heat stress in mice. For heat-stressed mice housed at a T_a below thermoneutrality (25°C; Ref. 11), recovery was characterized by a biphasic T_c response consisting of an initial hypothermia and a subsequent (~24 h) elevation in T_c. Although the magnitude and duration of hypothermia correlated with heat stress severity, the elevation in T_c ob-

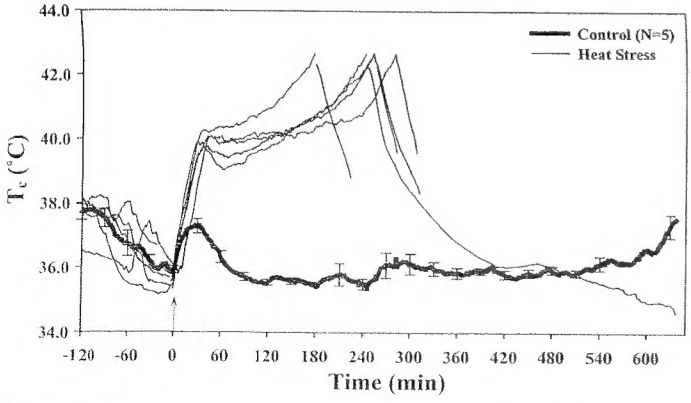

Fig. 4. T_c responses of C57BL/6J male mice heated (n = 5) at T_a = 39.5 ± 0.2°C to $T_{c,max}$ of 42.7°C compared with mean responses of controls (n = 5). Acclimation and recovery were at T_a = 30 ± 2°C. Arrow at *time 0* indicates start of heat stress. Each T_c trace ends at the time that the animal succumbed to heat stress.

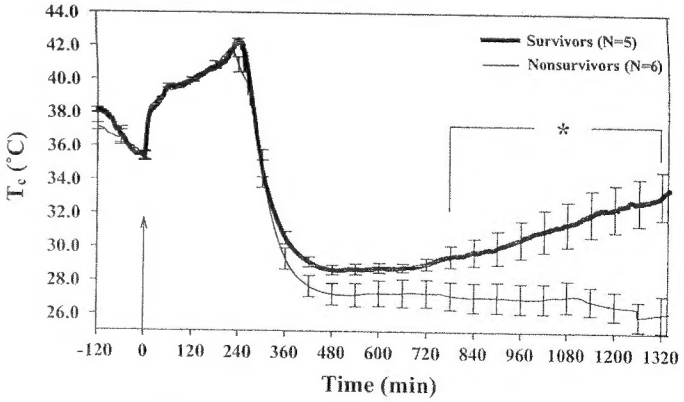

Fig. 5. T_c responses of survivors and nonsurvivors of C57BL/6J male mice heated at T_a = 39.5 ± 0.2°C to $T_{c,max}$ of 42.7°C. Acclimation and recovery were at T_a = 25 ± 2°C. Arrow at *time 0* indicates start of heat stress. Asterisk at 765 min denotes humane endpoint for study termination in a projected heat-stress nonsurvivor. Sample sizes (no. of mice) are indicated in parentheses. Significance set at $P < 0.05$.

served after transition out of hypothermia did not reflect differences in heat stress severity. When mice were housed and recovered at a T_a within their TNZ (30°C; Ref. 11), the development of hypothermia and the subsequent T_c elevation were prevented as survival rates were significantly decreased. These data suggest that ambient conditions can have a profound impact on heat stress recovery, and the development and transition out of hypothermia to produce an elevated T_c may be critical for survival in this species.

Before heat stress experimentation, mice were acclimated for at least 4 wk to the two common laboratory housing T_a levels of 25°C (A25 group, below the TNZ) and 30°C (A30 group, within the TNZ) for this species. Observations of A25 and A30 mice during their T_a acclimation period revealed virtually identical ultradian rhythms manifested as transient T_c spikes (~2°C inflections) that correlated with sleep arousal during their daytime or inactive period (data not shown). To minimize these potential influences, mice were included in a heat stress experiment only when daytime (between 0800 and 0900) or an inactive period baseline T_c of <36.0°C was observed. The establishment of this stable baseline T_c ensured that the environmental heat load (i.e., $T_{c,max}$ − baseline T_c) was virtually identical (~7.0°C) between mice tested in the three heat stress groups. It is important to note that previously described experimental heat stress experiments in rodents have been performed during the inactive period for rodents (e.g., Refs. 14, 26, 27, 31, 33, 34). It is not known whether T_c responses would differ if heat stress was initiated during the nighttime or active period, which corresponded to a baseline T_c of ~37°C in our study. Because this would reduce the environmental heat load by ~1°C, differences in the recovery T_c profile and survival rates would be expected for the three heat stress severities established in our model.

Our mouse heat stress profile had a triphasic pattern consisting of an initial steep rise in T_c, a hyperthermic plateau, and a final rapid progression to $T_{c,max}$ (Fig. 2). To minimize potential variability of heat stress profiles between groups, we tested equal numbers of mice from each shipment and tested each heat stress condition weekly. Despite this experimental design, the heating profiles of the extreme group showed less interindividual variability compared with the other heat groups (Fig. 2*C*). Although an analysis of time to $T_{c,max}$ indicated no significant differences between heat stress groups (Table 1), mice in the extreme group required significantly less time to reach 42.4°C (a T_c value that all groups attained, despite being heated to different $T_{c,max}$ values) compared with the other groups (data not shown). The three mice ($n = 1$ and $n = 2$ for moderate and extreme heat stress, respectively) that did not survive the first condition (heat stress) in the counterbalance design at A25 supported this trend. The reason for this difference in heating time is unknown but suggests that mouse variability in thermal tolerance is present in the absence of ancillary stresses and despite a protocol design to minimize interpopulation variability.

Previous studies have reported a range of minimal lethal temperatures (MLT) in heat-stressed rodents, ranging from 40.4 to 46.0°C (14, 26, 33), with the variability in these values likely a consequence of interanimal variability as well as differences in methodologies between studies. In our study, MLT was determined as 42.7°C, which corresponded to a 92% survival rate. This value is above the mouse MLT of 42.0°C reported by Wright (33), which reflected a 50% survival rate. In the study by Wright (33), mice were anesthetized and exposed to a chamber that was preheated to 41°C, which is more representative of a heat-shock paradigm compared with our present design. To more closely reflect what might be encountered in nature, we imposed heat stress by gradually increasing the T_{hs} to 39.5°C over an ~1-h time period. Our choice of a lower T_{hs} than Wright (33) was designed to provide a heating curve of sufficient duration to support future experimentation involving tissue and blood sampling profiles for potential heat injury mediators. To minimize confounders, our mice were not anesthetized and were acclimated to the incubator environment at their respective housing and recovery T_a for 24 h before experimentation. As such, our design of gradual heat stress exposure, with the minimization of ancillary stresses and the use of biotelemetry, perhaps provided a more accurate MLT estimation compared with previously described rodent heat stress models. In addition, the use of biotelemetry in our model represents advancement in design because it permitted an assessment of heat stress responses in undisturbed, unrestrained, conscious mice that were able to use behavioral and autonomic thermoeffector mechanisms to cope with prolonged exposure to a hot environment. The observation of wide variability in our model supports the hypothesis that thermal tolerance may be dependent on one or more presently unidentified physiological variables, rather than simply on methodological inconsistencies between studies.

Our experimental design required that heat-stressed mice be exposed to T_{hs} until they reached their preassigned $T_{c,max}$. As was the case for the single moderate heat stress lethality (Fig. 2*B*), those mice that were efficient thermoregulators, i.e., they effectively dissipated heat to the environment, were expected to require the longest time to reach $T_{c,max}$ and accrue the largest thermal areas. Because an elevation in total thermal area correlates with decreased survival rate (14), we expected more efficient thermoregulators to experience enhanced morbidity and mortality in our model. Surprisingly, the two animals with the lowest time to attain $T_{c,max}$ in the extreme group succumbed to heat stress (Fig. 2). Furthermore, an analysis of total and ascending thermal areas (used as a measure of thermal strain) revealed no significant differences between survivors and nonsurvivors of the extreme group. These data suggest that $T_{c,max}$ is a more accurate predictor of heat stress mortality than thermal area in our model.

An analysis of descending thermal area indicates that heat loss capacity during recovery has a significant impact on survival. As such, the presence of significantly larger descending thermal areas with increases in heat stress severity and during recovery at 30°C demonstrate the importance of the T_c/recovery T_a gradient for facilitation of heat dissipation. It appears from these results and the absence of circadian T_c differences between A25 and A30 mice that any heat stress advantage from A30 acclimation was not realized, since survival rate decreased to 0% at this T_a. We hypothesize that the reduced T_c/recovery T_a gradient fully accounted for the lack of hypothermia development and survival in A30 mice. Although we are unable to dissociate effects of recovery T_a from the T_a used for acclimation, we hypothesize that A25 moderate heat stress mice would also have experienced a significant reduction in survival rate if they were subjected to heat stress recovery at T_a of 30°C. These data suggest that, to avoid high heat stress

mortality, T_a should be maintained below the TNZ for the species under study such that hypothermia development is facilitated.

Mouse heat stress was followed by a period of hypothermia, which has been previously reported (31). However, unlike the earlier report, in the present study hypothermia occurred in the absence of anesthesia, a state known to induce hypothermia through inhibition of thermoregulatory mechanisms (13, 28, 30). Our observed heat stress-induced hypothermic response could be interpreted either as an unregulated event due to direct thermal damage to homeostatic sites (e.g., central nervous system) or an adaptive thermoregulatory survival mechanism. Wilkinson et al. (31) reported reduced intestinal damage and increased survival in hypothermic mice compared with those in which reductions in T_c are prevented. This suggests that heat stress-induced hypothermia is a protective, regulated phenomenon. Because HD and transition time were related to heat stress severity and in our study the absence of hypothermia transition (moderate and extreme groups) and hypothermia development (A30 mice) correlated with mouse mortality, there is the suggestion that hypothermia is a protective thermoregulatory event. Regulated hypothermia and unregulated hypothermia are not necessarily mutually exclusive events, since heat stress severity might dictate which is operative. In any case, because hypothermia was not universally protective in heat stress recovery (i.e., animals that did not arouse from hypothermia died), its status as a regulatory survival mechanism requires further study. A thermoregulatory profile of mice via a thermal gradient, for example, could allow mice to behaviorally select a wide range of T_a during heat stress recovery.

Interestingly, although ad libitum drinking water was available during recovery, mice did not immediately consume water after their removal from the heat (personal observations). It is unclear whether heat stress injury or the rapid development of hypothermia so compromised mechanisms of body water homeostasis that drinking behavior was impaired. Mice heated to their $T_{c,max}$ in the absence of drinking water lost ~13% of BW, which served as an indirect measure of dehydration. This BW loss was similar to that induced by 48 h of water deprivation in rats, which significantly impairs cardiovascular and thermoregulatory adjustments to heat stress (24). Dehydration induces hypothermia in amphibians (32), reptiles (18), and hamsters (15, 29), but its contribution to hypothermia development in heat-stressed mice has not been explored. Although it is interesting to speculate that dehydration facilitated development of the different phases of the thermoregulatory profile of A25 mice during recovery, the presence of similar dehydration levels despite differences in heat stress severity suggests that this variable is not a strong indicator of morbidity and mortality in our model. Clearly, to more fully define the contribution of dehydration to the observed T_c responses requires a study of mouse heat stress in the euhydrated condition.

After the hypothermia transition, an elevation in daytime T_c was apparent, irrespective of heat stress severity (Fig. 3). Extreme heat stress influenced a delayed arousal such that an elevation in T_c required longer to develop; once established, it was similar in magnitude to that of the mild and moderate A25 groups. Fever is defined as a regulated increase in the hypothalamic thermal set point. Scant evidence suggests its presence in clinical heat stress cases (1), although there is no evidence for its presence in experimental heat stress (31, 33). The absence of these data from experimental heat stress studies is most likely due to the inability to examine T_c in the unstressed state and throughout multiple circadian cycles using rectal probes or under restrained or anesthetized conditions (27, 31, 33, 34). Thus the presence of a feverlike state post-heat stress in our model was a novel observation that was observed via our use of biotelemetry. Evidence for the post-heat stress T_c elevations as a feverlike state is as follows: *1*) severe heat stress induces endotoxemia (4, 9, 12), *2*) circulating pyrogenic cytokines increase during endotoxemia/sepsis (7, 25) and heat stress (3, 5), *3*) the biphasic T_c profiles during mouse heat stress recovery (Fig. 2) were similar to those of sepsis and fever induced by endotoxemia or cecal ligation and puncture in mice (17, 22), and *4*) similar to LPS-induced fever responses (19), the feverlike phase post-heat stress was noted only during the day. Although fever's adaptive value during infection is recognized (16), its impact on heat stress recovery is unknown. It would be of interest to examine the effect of antipyretic treatment (e.g., aspirin) on the feverlike response to heat stress to determine whether it is a prostaglandin-mediated event. Because cytokines mediate thermoregulatory responses and influence mortality of mice subjected to septic or inflammatory stress (19, 20, 23), their study may be fruitful in revealing the importance of not only the post-heat stress feverlike state but the hypothermia phase that preceded it. The role of cytokines at several time points during heat stress recovery in A25 mice warrants further examination.

As heat stress severity increased, so did mortality (Table 1). Our modeling of extreme heat stress was necessary because mortality factors and interventions require identification and have not been previously established in the absence of ancillary stress. Through such modeling, humane endpoints were identified to minimize animal distress. The significant differences in HD and hypothermia transition time post-heat stress discriminated survivors from nonsurvivors to provide humane endpoints for study termination when extreme heat stress experiments are necessary. As indicated in our model, any mouse that did not recover from hypothermia by 765 min was not likely to survive. Thus any future experimentation with this model can minimize animal distress; that is, the study can be terminated in any mouse that has not begun transition out of hypothermia at this time point.

With avoidance of confounders such as anesthesia or restraint, study quality for morbidity and mortality factors of heat stress should improve. The present model could advance study quality because it employs biotelemetry to examine unrestrained, conscious mice during and after heat stress. After mice are subjected to different levels of heat stress severity, a biphasic T_c profile characterized by a T_a-dependent hypothermia and then what we hypothesize as a feverlike state were observed. Because the absence of hypothermia (A30) or prolonged hypothermia without recovery (A25 extreme heat stress) correlated with a significant reduction in survival rate, mouse heat stress recovery appeared linked to the development of this biphasic T_c response. Although this process may be unique to mice or animals of small body mass, elucidation of its mechanisms may reveal aspects of this recovery strategy that can be exploited for the prevention and treatment of human heat stress injury. As recently reviewed by Bouchama and Knochel (4), studies of genetically modified mice are required

to define human heat stress pathophysiology. The present model serves as an experimental paradigm to support such studies and to determine the physiological mechanisms behind mouse heat stress recovery.

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